Remarks

Claims 62 and 66-73 are pending in the subject application. Applicant acknowledges that claims 67-72 have been withdrawn from further consideration as being drawn to a non-elected invention. By this Amendment, Applicant has amended claim 62 and added new claims 74-83. Support for the amendments and new claim can be found throughout the subject specification and in the claims as originally filed (see, for example, page 2, line 21 through page 3, line 32; page 9, paragraph 1; page 10, lines 8-14; and page 11, paragraph 1). Entry and consideration of the amendments presented herein is respectfully requested. Accordingly, claims 62 and 66-83 are currently before the Examiner. Favorable consideration of the pending claims is respectfully requested.

Claims 62, 66 and 73 are rejected under 35 U.S.C. § 112, second paragraph, as indefinite. Applicant respectfully asserts that the claims as filed are definite and have addressed each rejection below.

Claims 62, 66 and 73 are indefinite in the recitation of the phrase "superagonistic signaling" since the term is not defined in the specification. The as-filed specification discusses the concept of superagonistic signaling at page 2, line 25 through page 3, line 32 and at page 11, paragraph 1 of the as-filed description. In these passages, both *in vitro* and *in vivo* superagonistic signaling are discussed. As noted in these passages, the concept relates to the binding of an antibody- or ligand-based agent to the PD-1 receptor within 75 Å of the cell surface, the binding of an antibody- or ligand-based agent to PD-1 in a fashion that brings the membranes of two cells within about 200 Å of each other or the surface of a cell within 200 Å of the surface of an immobilizing substrate to which an antibody is bound (*e.g.*, the surface of a plastic plate or a magnetic bead). Thus, one skilled in the art would have reasonably been apprised of the meaning of this term and reconsideration and withdrawal of the rejection is respectfully requested.

Claims 62, 66 and 73 are indefinite in the recitation of the phrase "membrane proximal region" because neither the reference point nor the degree of proximity have been defined. Applicant respectfully disagrees with this assessment. As indicated in the as-filed description at page 10, lines 8-15, the phrase "membrane proximal region" is defined as the extracellular portion of the receptor that is within 75 Å of a cell membrane. Thus, one skilled in the art would have reasonably been

apprised of the meaning of this term and reconsideration and withdrawal of the rejection is respectfully requested.

Claims 62, 66 and 73 are indefinite in the recitation of the word "only" as it is not known what it is intended to exclude and in the recitation of the word "equivalent" beeause the nature or degree of the requisite equivalence has not been defined. Applicant respectfully submits ahtat the term is clear in view of the teachings of the specification. Contrary to the assertion in the Office Action, one skilled in the art would have been apprised as to the meaning of the term "only". Partieularly, the as-filed specification clearly indicates (at page 11, lines 20-31) that one embodiment contemplates antibodies that do not exclusively bind to any or all of the sequcnces provided in Table 3. Applicant further submits that these issues are moot in view of the amendments made to the claims and reconsideration of the rejection is respectfully requested.

Claims 62, 66 and 73 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Office Action states that Applicant is not in possession of the claimed antibody, because Applicant is not in possession of the generically recited "cell surface receptor." Applicant respectfully asserts that there is adequate written description in the subject specification to convey to the ordinarily skilled artisan that they had possession of the claimed invention; however, it is respectfully submitted that this issue is now moot in view of the amendments made to the claims. Accordingly, reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, first paragraph, is respectfully requested.

Claims 62, 66 and 73 are rejected under 35 U.S.C. § 102(a) and 35 U.S.C. § 102(e) as anticipated by Hunig *et al.* (U.S. Published Application 2003/0166860). In addition, elaims 62, 66 and 73 are rejectcd under 35 U.S.C. § 102(b) as anticipated by Wood *et al.* (U.S. Published Application 2002/0160000). The Office Action indicates that Hunig *et al.* teach an antibody which binds to PD-1 epitope LAAFPEDRSQPGQDCR, which is identical in sequence to SEQ ID NO: 61 of the subject application. The Office Action notes that the Wood *et al.* publication teaches antibodies to PD-1 and, thus, anticipates the claimed invention. Applicant respectfully asserts that the cited referenees do not anticipate the claimed invention and note that SEQ ID NO: 61 is a

sequence that was not elected for examination in this matter at this time (SEQ ID NO: 44 having been eleeted). In the event that the Patent Office has expanded the examincd claims to include SEQ ID NO: 61, such an indication is requested. If the search and examination has been expanded to SEQ ID NO: 61, the indication of the allowability of the previously elected sequence (SEQ ID NO: 44) is respectfully requested since the search and examination could not have been expanded in that regard unless the elected invention was found to be allowable (see 37 C.F.R. §1.141).

Applieant submits that the Office Action has not established that the claimed invention is anticipated by the teachings of either Hunig *et al.* or Wood *et al.* In establishing the rejections of record, the Office Action is relying upon the doctrine of inherency, arguing that "the antibody taught by Hunig *et al.* has the same binding specificity as the instantly recited antibody, it inherently has the same functional properties, including the ability to induce superagonistic signaling" (see page 6 of the Office Action). It is well settled that in order for the Patent Office to establish a *prima facie* case of anticipation, each and every element of the claimed invention, arranged as required by the claim, must be found in a single prior art reference, either expressly or under the principles of inherency. *See generally In re Schreiber*, 128 F.3d 1473, 1477; *Diversitech Corp. v. Century Steps, Inc.*, 850 F.2d 675, 677-78 (Fed. Cir. 1988); *Lindemann Maschinenfabrik GMBH v. American Hoist and Derrick,* 730 F.2d 1452, 1458 (Fed. Cir. 1984). Additionally, the Patent Office cannot establish inherency merely by demonstrating that the asserted limitation is probable or possible. *In re Oerlich*, 666 F.2d 578, 581 (C.C.P.A. 1981). "Inherency may not be established by probabilities or possibilities. Thc mere fact that a certain thing may result from a given set of circumstances is not sufficient to establish inherency." *Scaltech Inc. v. Retec/Tetra, L.L.C.*, 178 F.3d 1378, 1384 (Fed. Cir. 1999). *See also Cont'l Can Co. USA, Inc. v. Monsanto Co.*, 948 F.2d 1264, 1268-69 (Fed. Cir. 1991).

Turning first to the teachings of Hunig *et al.*, Applicant notes that the antibodies in that reference were generated against "peptides, protein according to the invention or a mimicry compound thereto" (see paragraph 33). It is further noted that a "peptide or protein according to the invention" is defined in paragraph 30 as the C'-D peptides in Figure 7 to which one or two additional amino acids can be added to the 3' or 5' end. Other peptides of the invention are subsequences of the boxed sequences identified in Figure 7. Thus, it is clear that the antigen uscd to generate

Found. For Med. Educ. & Research, 346 F.3d 1051, 1054, 68 USPQ2d 1373, 1376 (Fed. Cir. 2003). Additionally, "[i]nherency may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient to establish inherency." *Scaltech Inc. v. Retec/Tetra, L.L.C.*, 178 F.3d 1378, 1384 (Fed. Cir. 1999). *See also Cont'l Can Co. USA, Inc. v. Monsanto Co.*, 948 F.2d 1264, 1268-69 (Fed. Cir. 1991).

Thus, while Wood *et al.* teach the production of antibodies that bind to PD-1, the reference is limited to teaching the production of antibodies to PD-1 and provides no teaching as to how one skilled in the art is to obtain superagonistic antibodies. Applicant further submits that the reference also fails to provide any recognition that superagonistic antibodies could be produced and there is no teaching in the reference of superagonistic antibodies. Thus, Wood *et al.* fail to provide adequate teachings to enable the production of superagonistic antibodies, fail to teach superagonistic antibodies and cannot anticipate the claimed invention. As also noted above, the possibility or probability the production of superagonistic antibodies may result from the teachings of Wood *et al.* is not sufficient to establish inherency. Accordingly, reconsideration and withdrawal of the rejections under 35 U.S.C. § 102(b) is respectfully requested.

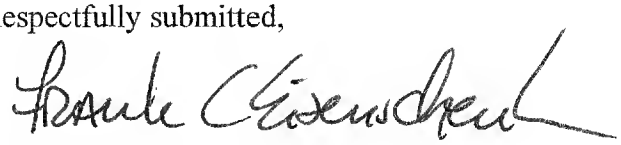
It should be understood that the amendments presented herein have been made solely to expedite prosecution of the subject application to completion and should not be construed as an indication of Applicant's agreement with or acquiescence in the Examiner's position. Applicant expressly reserves the right to pursue the invention(s) disclosed in the subject application, including any subject matter canceled or not pursued during prosecution of the subject application, in a related application.

In view of the foregoing remarks and amendments to the claims, Applicant believes that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Applicant invites the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,



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Resistance of Human Immunodeficiency Virus Type 1 to Neutralization by Natural Antisera Occurs through Single Amino Acid Substitutions That Cause Changes in Antibody Binding at Multiple Sites

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The ability of human immunodeficiency virus type 1 (HIV-1) to replicate in the presence of strong immune responses to the virus may be due to its high mutation rate, which provides envelope gene variability for selection of neutralization-resistant variants. Understanding neutralization escape mechanisms is therefore important for the design of HIV-1 vaccines and our understanding of the disease process. In this report, we analyze mutations at amino acid positions 281 and 582 in the HIV-1 envelope, where substitutions confer resistance to broadly reactive neutralizing antisera from seropositive individuals. Neither of these mutations lies within an antibody-binding site, and therefore the mechanism of immune escape in both cases is by alteration of the shape of the envelope proteins. The conformation of the CD4-binding site is shown to be critical with regard to presentation of other discontinuous epitopes. From our analysis of the neutralization of these variants, we conclude that escape from polyclonal sera occurs through alterations at several different epitopes, generally resulting from single amino acid substitutions which influence envelope conformation. Experiments on a double mutant showed that the combination of both mutations is not additive, suggesting that these variants utilized alternate pathways to elicit similar alterations of the HIV-1 envelope structure.

Studies on the neutralization of human immunodeficiency virus type 1 (HIV-1) by monoclonal antibodies have identified a number of targets on the HIV-1 envelope proteins gp120 and gp41 as neutralizing sites. The best characterized of these are the principal neutralization determinant or V3 loop (8, 10, 14, 15, 19, 32, 38) and the CD4-binding site (for a review, see reference 39). Additional sites have been identified in the C2 (13) and V2 (7, 9, 12, 24) regions of gp120, as well as in gp41 (2, 3, 28). Other, conformational sites have been described previously (40, 44) but are not yet fully characterized. The progression of HIV-1 infection from the asymptomatic stage to AIDS is thought to be associated with outbreaks of "miniviremia" (30) due to the evolution of neutralization-resistant virus strains during the course of the infection (1). On the basis of these observations, it has been suggested that protection against HIV-1 will best be achieved by a vaccine or other immunotherapeutic agent which elicits broadly reactive neutralizing antibodies against epitopes of HIV-1 that show minimal variability (6, 11, 20, 30, 34). There is, however, minimal information on the nature of the variants responsible for the outbreaks of miniviremia and therefore no basis for the design of appropriate vaccines or immunotherapeutics with this model.

One way to elucidate immune escape mechanisms and identify sites which elicit broadly reactive neutralizing antibodies is to analyze in vitro variants immune selected with heterotypic antisera. We have previously used this approach (37) to identify two separate single amino acid substitutions, one (A281V) in gp120 (50) and the other (A582T) in the gp41 transmembrane protein of HXB2 (35), which confer neutralization resistance to a significant proportion of sera from seropositive individuals which neutralize the parental virus (50, 52). Neither of these mutations occurs in an antibody-binding site (50, 52). Analysis of the A281V variant could not identify an individual neutralizing site that was affected by the mutation (50), but the A582T mutation clearly affected the ability of antibodies to the conformation-dependent CD4-binding site of gp120, exemplified by the monoclonal antibody F105 (33, 46), to neutralize the variant virus (16, 43). The A582T variant was also shown to be resistant to monoclonal antibody 48d (43), whose binding to viral envelope is enhanced in the presence of CD4 (45). It has also been shown that the A582T variant reverts to a nonresistant phenotype in the absence of selective pressure (41).

It is notable that in both cases, when an antiserum rather than a monoclonal antibody was used to select neutralization-resistant variants of HIV-1, the mutations that appeared were not in known neutralizing-antibody-binding sites as has been reported, for example, for escape mutants selected with monoclonal antibodies specific for the V2 (54) or V3 (21) loop regions. Instead, variants selected in this way have evolved mutations that cause structural changes which affect the binding of neutralizing antibodies to gp120. This is not surprising since among the neutralizing antibodies elicited in in vivo infection, those directed to conformational epitopes are highly prevalent (23). While some in vivo selection has involved linear neutralization sites (29, 47), immune system pressure is just as likely to select variants with mutations outside discrete sites. In the A582T variant, a change in gp41 led to an alteration in an area of gp120 closely congruent with the CD4-binding site such that some antibodies that would inhibit the binding of gp120 to

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CD4 no longer recognized gp120. Analyses of the A281V variant have not revealed an equivalent resistance to any monoclonal antibody.

In this report, we describe our attempts to further characterize the mechanism by which these two variants became resistant to neutralization. We examined the neutralization resistance of the immune system-selected variants, A582T and A281V, as well as the naturally occurring variants, A281T and A281I, to a broader range of HIV-1-neutralizing monoclonal antibodies and human sera. We reexamined the neutralization resistance of the A582T variant to human immune sera in view of its previously unrecognized propensity for reversion (41). Finally, we assessed the effects on HIV-1 neutralization of combining both escape mutations (A281V and A582T) in a single virus.

All the variants tested showed some resistance to neutralization by monoclonal antibodies to more than one epitope. From this, we surmise that escape from neutralizing antisera may occur not primarily by alteration of a single epitope but through mutations that affect the overall structure of gp120 and influence the binding of different antibodies to numerous sites on the protein. Such a process occurring in vivo could explain, at least in part, the general resistance of primary isolates to neutralization in vitro.

MATERIALS AND METHODS

Cell culture and virus strains. Cos-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 25 μg of gentamicin per ml, 2 mM L-glutamine, and 10% fetal bovine serum, passaged, and subcultured by trypsinization by using standard techniques. H9 cells were grown in RPMI 1640 medium supplemented with 1 to 2 mM L-glutamine, 10% fetal bovine serum and either 25 μg of gentamicin per ml or 100 U of penicillin per ml plus 100 μg of streptomycin per ml. The viral strain HXB2 was expressed from a plasmid containing a full-length infectious molecular clone (pHXB2) (4) by electroporation of Cos-1 cells and subsequent transmission to H9 cells by coculture as previously described (41, 50). Molecularly cloned variant virus constructs derived from HXB2 were expressed and cultured as described for HXB2.

Construction of variant viruses. The construction of HXB2 variant viruses containing the mutation A582T or A281V has been described previously (35, 50). The mutations A281I and A281T were generated by PCR mutagenesis (48, 49), and the resulting variant *SalI-PvuII* fragments were subcloned with the *PvuII-BamHI* fragment of HXB2, directly into pHXB2. The A281V/A582T double mutant was constructed by cocloning the *SalI-NheI* fragment of the A281V variant and the *NheI-BamHI* fragment of the A582T variant into a *SalI-BamHI*-digested pHXB-2 vector preparation.

Immunologic assays. (i) Neutralizing-antibody assay and monoclonal antibodies. Neutralizing-antibody titers of HIV-1-positive human sera and monoclonal antibodies were determined as previously described (36) with fresh supernatant media from H9 cells infected with HXB2 or variant constructs as a source of virus. Neutralizing-antibody titers were defined as the reciprocal of the serum dilution or the monoclonal antibody concentration at which infectivity levels were 60% of control levels following normalization of the data to control values. The monoclonal antibodies used included an anti-V2-loop antibody, 52-684-238 (24), generously provided by W. Gerard Robey, Abbott Laboratories; three anti-V3-loop antibodies, 0.5β (19) and IIIB-V3-13 (17), both obtained through the AIDS Reference and Reagent Repository, NIAID, and M77 (31), generously provided by Fulvia di Marzo Veronese, National Cancer Institute; two anti-CD4-binding-site antibodies, F105 (33, 46), obtained through the AIDS Reference and Reagent Repository, and 120-IBI, obtained from Virus Testing Systems Corp., Houston, Tex.; two CD4-binding-sensitive antibodies, 17b (45) and 48d (45); and an anti-gp41 antibody, 41-2F5 (28), obtained from Virus Testing Systems Corp. A variant virus was judged to be neutralization resistant if at least fivefold more antibody was necessary for its neutralization compared with the amount needed for neutralization of the parental virus, HXB2.

(ii) Binding of 17b and 48d to HXB2 and variant envelopes in supernatant media by ELISA. The amount of gp120 in tissue culture supernatants of H9 cells infected with HXB2 or variant viruses was determined by a gp120 antigen capture assay (Intracel Corp., Cambridge, Mass.). The concentration of supernatant gp120 was similar for all viruses, ranging from 5 to 7 ng/ml. The binding of monoclonal antibodies 17b and 48d to gp120 was determined by enzyme-linked immunosorbent assay (ELISA) essentially as previously described (22, 45). Briefly, 100 μl of a 10-μg/ml solution of sheep anti-gp120 (D7324; International Enzymes Inc., Fallbrook, Calif.) in carbonate-bicarbonate buffer (pH 9.6) was adsorbed overnight at 4°C onto wells of Immulon I plates (Dynatech Ltd., Chantilly, Va.). The plates were washed three times with water and blocked for 30 min at room temperature with 5% bovine serum albumin in phosphate-buffered saline (PBS). The plates were again washed with water, and 100 μl of supernatant media containing gp120 with and without 1 μg of recombinant soluble CD4 (sCD4; American Biotechnologies Inc., Cambridge, Mass.) per ml was added. Following incubation at 37°C for 1 h, the plates were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween). Then 100 μl of 10-fold serial dilutions of 48d and 17b monoclonal antibodies in PBS containing 1% normal goat serum was added to the wells and incubated for 1 h at 37°C. Human immunoglobulin G served as a control. The plates were washed three times with PBS-Tween, 100 μl of goat anti-human immunoglobulin G-peroxidase (Kirkegaard and Perry, Inc., Gaithersburg, Md.) appropriately diluted in PBS–1% normal goat serum was added, and the mixture was incubated for 1 h at room temperature. After washing with PBS-Tween, the plates were developed for 20 min with substrate solution containing 0.5 mg of *o*-phenylenediamine dihydrochloride per ml and 0.03% hydrogen peroxide in citrate buffer (pH 5.0). The reaction was stopped by the addition of 50 μl of 4 N sulfuric acid, and the A_{492} was read.

Binding of 17b and 48d to H9 cells infected with HXB2 and variant viruses. Virus-infected cells were washed twice in PBS and adjusted to 5×10^6 cells per ml in PBS. Then 100 μl of cell suspension was added to wells of 96-U-well plates, the plates were centrifuged at 1,500 rpm in a Sorvall H1000B rotor for 10 min, and the PBS was aspirated. A 50-μl volume of fivefold serial dilutions of 17b and 48d antibodies (beginning at 10 μg/ml) with and without 50 μl of recombinant sCD4 (10 μg/ml) was added to each well, and the wells were incubated at 37°C for 30 min. Human immunoglobulin G and the fluorescein isothiocyanate conjugate were used as controls. After washing of the plates twice with cold PBS containing 0.1% sodium azide (PBS-azide), 50 μl of appropriately diluted goat anti-human immunoglobulin G-fluorescein isothiocyanate (Biosource International, Camarillo, Calif.) was added to the wells. After another 30-min incubation at room temperature, the cells were washed twice with PBS-azide, suspended in 200 μl of 1% paraformaldehyde, and analyzed on the FACScan.

RESULTS

The A582T variant of HIV-1_{HXB2} is resistant to most natural antisera. The A582T variant of HIV-1_{HXB2} was resistant to neutralization, as defined by a decrease in neutralizing titer of fivefold or more compared with the neutralization of HXB2, by 86% (19 of 22) of sera tested which were able to neutralize HXB2 (Table 1). The three sera with which variant A582T did not meet the resistance criterion (WN529, W0426, and W0745) nevertheless neutralized variant A582T with titers two- to fourfold lower than those for HIV-1_{HXB2}. This figure (86%) is higher than the previously reported resistance of variant A582T to neutralization by human sera (52) because it was not possible to culture the variant continuously in the selecting serum and we were previously unaware of the evolution of phenotypic revertants (41) in the absence of continuous immune pressure. The experiments in this study were performed with stocks of variant A582T obtained within 2 to 4 weeks of transfecting the Cos-1 cells with the variant virus constructs to ensure that reversion was minimized. Thus, not only was the A582 variant resistant to most of the human sera tested, but also the mean neutralizing titer of human sera for variant A582T was more than 10 times lower than that of HXB2 (26 compared with 371), illustrating the profound effects conferred by this mutation on neutralizability.

Variants A281I and A281T share the resistance of A281V. Amino acid substitutions for alanine at position 281 found among clade B HIV-1 isolates include valine (12%), threonine (5%), and isoleucine (2%) (50). The resistance of these variants to neutralization by human sera was less pronounced than the resistance of A582T (Table 1). All three variants tested were resistant to neutralization by the serum used in the selection of the A281V variant (50) and generally were resistant to approximately 25% of the sera able to neutralize HXB2. Interestingly, some sera showed increased neutralization sensitivity on 2 or more of the 281 variants (W0747, RT), although these increases were not significant. The mean neutralization titer for variant A281V was almost four times lower than for HXB2 (102 compared with 371). Variants A281I and A281T showed similar decreases in mean neutralization titer (98 and

TABLE 1. Neutralization of variant viruses by human sera

Serum	Titer of virus:					
	HXB2	A582T	A281V	A281I	A281T	A582T/A281V
WN510	260	<10	65	45	40	<10
WN512	1,705	45	100	295	195	60
WN524	50	<10	20	35	55	15
W0380	110	15	65	25	50	30
W0395	90	<10	<10	30	40	15
W0747	70	<10	95	205	85	30
W0925	1,000	130	55	40	125	115
W6235	120	<10	60	40	25	45
RT	175	30	255	345	150	80
0731	>605	45	175	NT[a]	NT	65
WN402	425	10	140	NT	NT	100
W0920	>635	60	470	NT	NT	115
W0919	450	35	150	NT	NT	95
W0885	1,175	35	155	NT	NT	90
W9974	345	40	180	NT	NT	60
W9966	60	<10	30	NT	NT	70
W0378	65	<10	30	NT	NT	10
WN526	435	10	60	NT	NT	145
W9969	190	20	80	NT	NT	95
WN529	40	<10	<10	30	20	25
W0426	125	55	35	60	30	35
W0745	35	15	25	20	15	20
Mean neutralizing titer ± SEM	371 ± 92	26 ± 6	102 ± 22	98 ± 33	69 ± 17	60 ± 9
% of neutralizing sera[b]	100	14	77	75	75	59

[a] NT, not tested.
[b] For variant viruses, resistance to neutralization is defined as requiring at least a fivefold-higher serum concentration than for HXB2.

69, respectively, compared with 315 for HXB2 on the same set of sera). These observations suggest that the behavior of the variants with I and T at position 281 is similar to that of the V variant, rather than of the prevalent A at this position.

Variants with mutations at position 281 show resistance to neutralization at more than one epitope. Previously, we reported that the A281V variant was sensitive to neutralization by soluble CD4 and CD4-binding-site antibodies and that slight increases in the amounts of V3-loop-specific antibodies were necessary for its neutralization (50). These observations were confirmed here (Table 2). Further studies to characterize the A281I and A281T variants, however, showed they possessed a slightly more resistant phenotype than A281V and that all three variants exhibited neutralization resistance to antibody 48d, whose binding is reported to be enhanced in the presence of soluble CD4 (45). The amount of 48d antibody required to neutralize all three of the variants with mutations at position 281 ranged from 8- to 15.5-fold higher than that for HXB2. Greater concentrations of monoclonal antibody 17b, another CD4-binding-sensitive antibody (45), were necessary to neutralize the three variants compared with HXB2, but a fivefold difference was not attained. Variant A281V was pre-

TABLE 2. Neutralization of HXB2 and variant viruses by monoclonal antibodies

Antibody	Mean neutralizing-antibody titer ± SEM for[a]:					
	HXB2	A582T	A281V/A582T	A281V	A281T	A281I
V2 region						
52-684-238	5.40 ± 1.6	6.5 ± 2.2 (1.2)	10.7 ± 2.7 (2.0)	10.1 ± 3.0 (1.9)	14.6 ± 3.4 (2.7)	12.5 ± 2.2 (2.3)
V3 region						
0.5β	0.33 ± 0.04	0.99 ± 0.22 (3.0)	0.98 ± 0.27 (3.0)	0.96 ± 0.24 (2.9)	2.5 ± 0.62 (7.6)	2.8 ± 1.12 (8.5)
IIIB-V3-13	4.3 ± 2.9	3.4 ± 1.5 (0.8)	3.2 ± 1.8 (0.7)	7.5 ± 1.3 (1.7)	8.5 ± 1.5 (2.0)	4.4 ± 2.8 (1.0)
M77	763 ± 200	658 ± 218 (1.2)	600 ± 440 (1.3)	467 ± 24 (1.6)	620 ± 300 (1.2)	277 ± 102 (2.8)
CD4-binding site						
F105	1.4 ± 0.45	>10.9 ± 0.88 (7.8)	12.4 ± 4.2 (8.9)	1.0 ± 0.61 (0.7)	2.8 ± 1.3 (2.0)	2.8 ± 0.92 (2.0)
120-IBI	11.8 ± 4.8	>20 ± 0 (1.7)	13.8 ± 6.2 (1.2)	11.2 ± 4.8 (0.9)	11.5 ± 4.7 (1.0)	6.4 ± 2.8 (0.5)
CD4-binding sensitive						
17b	2.9 ± 2.1	19.3 ± 0.67 (6.7)	14.4 ± 2.8 (5.0)	9.2 ± 5.4 (3.2)	12.3 ± 0.88 (4.2)	8.5 ± 1.8 (2.9)
48d	0.4 ± 0.13	14.3 ± 2.9 (35.8)	14.3 ± 1.5 (35.8)	4.4 ± 2.8 (11.0)	6.2 ± 3.2 (15.5)	3.2 ± 1.0 (8.0)
gp41						
41-2F5	0.35 ± 0.23	1.4 ± 0.49 (4.0)	1.6 ± 0.91 (4.6)	0.46 ± 0.17 (1.3)	1.0 ± 0.31 (2.9)	0.84 ± 0.36 (2.4)

[a] Neutralizing-antibody titer is expressed in micrograms per milliliter except for M77, which is the reciprocal dilution. The titer relative to HXB2, given in parentheses, refers to the fold increase in amount of antibody needed to neutralize each variant compared to HXB2.

viously shown to be neutralization sensitive to two monoclonal antibodies to the CD4-binding site, F105 and 120-IBI (50). Here we show that the A281I and A281T variants are also sensitive to these antibodies (Table 2). Slightly more V2 region monoclonal antibody, 52-684-238, was required to neutralize the A281T and A281I variants than to neutralize HXB2. Similarly, A281I and A281T exhibited increased resistance to neutralization by anti-V3-loop monoclonal antibody 0.5β but not by two other anti-V3-loop monoclonal antibodies, M77 and IIIB-V3-13. A monoclonal antibody that recognizes a conserved gp41 epitope, 41-2F5, neutralized A281V as readily as HXB2, and only modest increases in antibody concentration were required for neutralization of A281I and A281T.

Variant A582T shows greatly increased resistance to neutralization by CD4-binding-site antibodies and antibodies whose binding is enhanced in the presence of sCD4. We previously reported that the A582T variant was resistant to neutralization by antibodies directed to the conformationally determined CD4-binding site but was unaffected by V3-specific antibodies (16, 35). We further investigated the neutralization phenotype of this variant with the panel of monoclonal antibodies shown in Table 2. The results confirmed that this variant shows greatly increased resistance to neutralization by some antibodies that recognize the conformational CD4-binding site as well as CD4-binding-sensitive antibodies. The greatest resistance was seen with monoclonal antibody 48d, for which the relative amount of antibody required to neutralize the A582T variant was 35.8-fold higher than that required to neutralize HXB2 (Table 2). Resistance to this monoclonal antibody confirms the previous result of Thali et al. (43). The magnitude of the resistance of A582T was not the same for both CD4-binding-sensitive antibodies or for antibodies targeting the CD4-binding site. Monoclonal antibody 17b showed only moderate resistance, for example, while 120-IBI showed no effect.

The A582T variant was sensitive to the V2-region monoclonal antibody 52-684-238, while anti-V3-loop monoclonal antibody 0.5β showed a slight increase in the concentration required to neutralize A582T. Similarly, the anti-gp41 monoclonal antibody 41-2F5 neutralized A582T less well than it neutralized HXB2, although a fivefold difference in titer was not achieved. The two other anti-V3-loop monoclonal antibodies tested, M77 and IIIB-V3-13, neutralized variant A582T with titers similar to those for HXB2.

Combining neutralization resistance mutations A281V and A582T does not increase resistance to neutralization. Tested against a panel of human sera, the virus containing both the A582T and A281V mutations remained resistant to the serum used for the selection of the A281V variant (W0925) but not to the serum used for the selection of the A582T variant (RT) (Table 1) as judged by our fivefold-difference criterion. Considering the polyclonal nature of the human sera, this result is not surprising, because the RT serum neutralized A281V with higher titer than it neutralized HXB2. Overall, the double variant was resistant to neutralization by 41% of the sera tested, demonstrating significantly more resistance to this panel of sera than the A281V alone but considerably less resistance than the A582T variant. The mean neutralizing titer showed the same effect; the mean titer for the A582T-A281V double variant was 60 compared with 26 for A582T, 102 for A281V, and 371 for HXB2 on the same panel of sera. Thus, using polyclonal human sera, the A281V mutation appeared to modulate the effects of the A582T change. That the A281V mutation does not completely compensate for the A582T alteration, however, is shown by the studies with the panel of

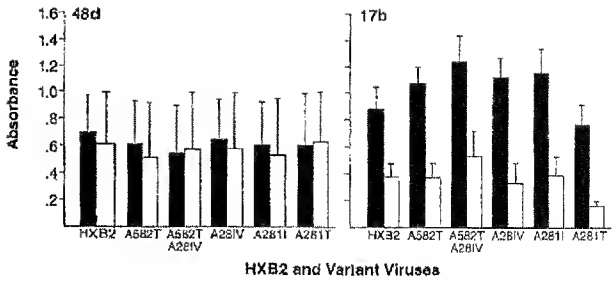

FIG. 1. Binding of monoclonal antibodies 17b and 48d to the envelope proteins in culture supernatants of HXB2- and variant virus-infected cells in the presence and absence of sCD4. Binding was assessed by using monoclonal antibodies at 100 ng/ml. Results are expressed as mean absorbance ± standard error of the mean (SEM). Solid bars, sCD4 present; open bars, sCD4 absent.

monoclonal antibodies (Table 2). In every case, the double variant showed almost identical behavior to variant A582T.

Effect of CD4 binding on neutralization of variant viruses. Since the A582T and the three 281 variants showed significant resistance to neutralization by monoclonal antibody 48d and a decreased neutralization titer with 17b, binding studies were carried out to probe the basis for the resistance. Specifically, we examined both the effect of sCD4 on antibody binding and the effect of monomeric versus cell-associated envelope on antibody binding to the variant virus envelopes. In contrast to earlier studies (45), we found that the binding of 17b to HXB2 gp120 was more influenced by sCD4 than was that of 48d, approximating a twofold enhancement (Fig. 1). Comparing HXB2 with the variant viruses, no significant differences in the binding of either 17b or 48d were observed. These data suggest that the increases in neutralization resistance seen with the variant viruses compared with HXB2 cannot be explained by changes in the accessibility of 17b and 48d epitopes on monomeric gp120. To confirm this, the affinities of 17b and 48d for monomeric gp120 of the different viruses were measured. No significant differences in binding affinity of 48d between HXB2 and the variant viruses were seen (Fig. 2). The relative affinity of 17b for gp120 was observed to be lower than that of 48d.

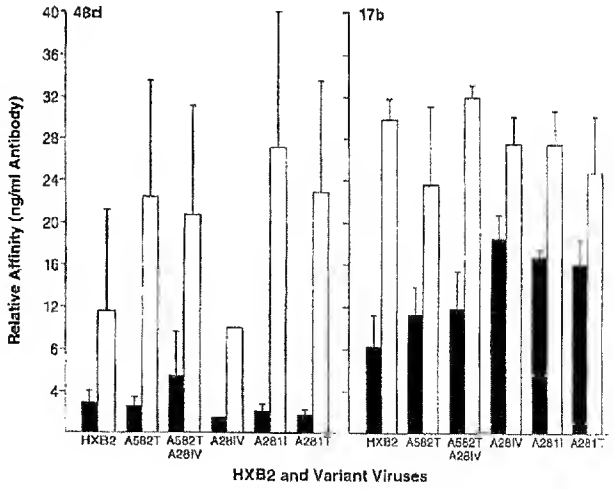

FIG. 2. Relative binding affinities of monoclonal antibodies 48d and 17b to envelope proteins in culture supernatants of HXB2- and variant virus-infected cells. Results are expressed as the mean relative affinity ± SEM. Relative affinity is defined as the antibody concentration (in nanograms per milliliter) at which a 50% reduction in absorbance was observed. The relative affinity of variant A281V with monoclonal antibody 48d was determined only twice, so the SEM is not given. Solid bars, sCD4 present; open bars, sCD4 absent.

TABLE 3. Binding of monoclonal antibodies 48d and 17b to cells infected by HXB2 and variant viruses in the presence or absence of sCD4

Virus	Staining of cells with monoclonal antibody[a]:							
	48d				17b			
	+sCD4		−sCD4 (% of positive cells)	Fold increase with sCD4	+sCD4		−sCD4 (% of positive cells)	Fold increase with sCD4
	MFI[b]	% of positive cells			MFI	% of positive cells		
HXB2	194	72	55	1.3	273	91	86	1.1
A582T	113	43	15	2.9	98	54	40	1.4
A281V	122	45	14	3.2	96	51	36	1.4
A582T/A281V	98	39	13	3.0	100	52	36	1.4
A281I	84	33	9	3.7	123	50	35	1.4
A281T	90	32	11	2.9	152	57	42	1.4

[a] Expression of envelope protein on the surface of H9 cells infected with the various viruses was equivalent as judged by staining with a pool of human sera possessing high-titer antibodies to HIV-1 envelope. Results represent the means of duplicate determinations.
[b] MFI, mean fluorescence intensity.

Moreover, the relative affinity of 17b for the 281 variants was approximately twofold lower than that for HXB2. This difference may contribute in part to the increase in antibody concentration necessary to neutralize these variants compared with HXB2, but it cannot account for the full magnitude of the difference (Table 2).

Because the decreased sensitivity of all the variant viruses to neutralization by 17b and 48d could not be explained by alterations in epitope accessibility or binding affinities of the antibodies for monomeric gp120, we investigated the interaction of the antibodies with native envelope expressed on the surface of infected cells. The enhanced binding of 17b and 48d in the presence of sCD4 previously reported (45) was not seen here. The enhancing effects of sCD4 are subtle, however, and the different results probably reflect experimental variables, such as pH and ionic strength, which can influence envelope conformation, as well as differences in envelope expression attributable to the COS system (45) versus the infected H9 cell system studied here. In any case, both antibodies showed lesser recognition of all variant viruses than of HXB2 in the presence of sCD4 (Table 3). In the absence of sCD4, all of the variants exhibited a three- to fourfold decrease in the binding of 48d compared with only a 1.3-fold decrease for HXB2. In contrast, sCD4 had little influence on the binding of 17b to the variant virus cell surface-expressed envelopes.

We also determined the affinities of 48d and 17b for HXB2 and the variant virus envelopes in the cell-associated form. The results of these assays showed no significant differences between HXB2 and the variants (Table 4). However, the affinity of 17b was approximately fivefold higher for cell-associated gp120 than was that of 48d. This reverses the relative affinities of the two antibodies with respect to those seen for monomeric gp120 (Fig. 2) and suggests that 17b, but not 48d, recognizes multimeric (cell-associated) gp120 much more strongly than it recognizes monomeric gp120.

TABLE 4. Relative binding affinities of antibodies 48d and 17b for cells infected by HXB2 and variant viruses[a]

Virus	Affinity of antibody:	
	48d	17b
HXB2	1.1	0.24
A582T	1.8	0.35
A281V	1.8	0.29
A582T/A281V	1.4	0.40
A281I	1.6	0.35
A281T	1.4	0.24

[a] Relative affinity is defined as the antibody concentration at which a 50% reduction in mean fluorescence intensity was observed. Results represent the means of duplicate determinations.

DISCUSSION

HIV-1 causes a chronic viral infection that progresses to AIDS 5 to 10 years after infection in the majority of cases. Most data suggest that few, if any, infected individuals either clear the infection or enter a phase of viral latency, as determined by the observed lack of seroreversion (loss of serum antibody titer, with no detectable viral RNA in plasma). Most infected individuals show increasing antibody titers in serum and increasing numbers of proviral genomes in peripheral blood lymphocytes with time. Both of these observations indicate a persistent active viral infection despite the presence of high neutralizing-antibody titers. The most likely explanation for these observations is that viral replication continues in the presence of high-titer neutralizing antibodies through the evolution of neutralization escape variants. The evolution of these mutants in the presence of natural human sera is therefore potentially of great importance in understanding the disease process underlying AIDS.

To study this process, we developed an in vitro system and subsequently identified two single amino acid substitutions (A281V and A582T) that resulted in the neutralization resistance of the variant virus to some HIV-1-positive human sera. In this study, we have investigated three issues relevant to these variants and to the interaction between HIV-1 and host humoral immune responses in general. First, we have determined the neutralization resistance of other naturally occurring variants with mutations at position 281 to assess their potential role in the in vivo immune escape and disease progression. Second, we have explored the route of immune escape used by variants at positions 582 and 281. Finally, we have combined the two neutralization escape substitutions in the same virus to assess the probability that HIV-1 might be able to utilize combinations of escape mutations to further avoid neutralization.

Two other naturally occurring variants with mutations at position 281 (A281I and A281T) exhibited the same pattern of resistance to a panel of human sera as did the original variant, A281V (Table 1). There was no evidence for greater resistance to neutralization or resistance to neutralization by additional sera. From these observations, we conclude that the substitution of either V, T, or I for the dominant A at position 281 in

gp120 may give rise to the same neutralization-resistant phenotype. This is in contrast to our previous analysis of variants with mutations at position 582, for which all but the A582T variant were either sensitive to neutralization or nonviable (52).

Our investigation of the means of neutralization resistance of the position 281 variants by using a panel of monoclonal antibodies did not identify a single epitope at which the variants were resistant to neutralization. Instead, resistance to antibodies recognizing different epitopes, in particular, V3 monoclonal antibody 0.5β and CD4-binding-sensitive monoclonal antibody 48d, was noted. From these observations, we concluded that the resistance of position 281 variants to neutralization was probably mediated by envelope conformational changes leading to alterations in several neutralizing-antibody-binding sites.

The resistance of the A582T variant to 86% of the human sera tested emphasizes the impact of a single amino acid substitution on the CD4-binding-site region, where a change in conformation can lead to widespread neutralization resistance involving antibodies directed to several envelope regions. The double mutant, which contained both the A281V and the A582T substitutions, exhibited basically the same pattern of resistance to human HIV-1-positive sera as did the A582T variant, but the degree of resistance was somewhat lower. Its pattern of resistance to the monoclonal antibodies was also very similar to that of A582T. These observations suggest that effects of A582T and A281V are not additive, but, rather, that the A281V substitution can reduce the effect of the A582T substitution on neutralization by polyclonal sera.

The greatest degree of neutralization resistance seen for the A582T and 281 variants occurred with monoclonal antibody 48d. However, resistance to the similar CD4-binding-sensitive monoclonal antibody, 17b, was not equivalent. Because of this, as well as previously reported data that the A281V variant was not resistant to 48d or 17b (45), we investigated the dependence of both antibodies on two factors thought to influence HIV-1 neutralization: the exposure of sites upon CD4 binding and the requirement for multimers of gp120 in order for high-affinity binding to occur. Our initial assessments of binding to monomeric gp120 by ELISA suggested that there was little or no dependence of 48d binding on CD4 and only a slight improvement in the binding of 17b in the presence of CD4 (Fig. 1). However, when the binding of these antibodies to infected cells was assessed, the dependence on CD4 of 48d binding to all the variant viruses became evident, although this was less evident for HXB2 (Table 3). No difference was seen with 17b. These data suggest that the resistance to 48d of all the variants tested is due to reduced availability of the epitope recognized by this antibody, particularly in the absence of CD4. Equally interesting is the observation that the affinity of 17b for cell-associated gp120 was measured at between 0.24 and 0.4, depending on the variant assessed (Table 4). For free gp120, the equivalent values were 8 to 18 (Fig. 2). This approximately 40-fold range presumably represents the difference in affinity of 17b for monomeric gp120 versus multimeric cell- or virion-associated envelopes, with the multimeric form being more strongly recognized. No equivalent change in affinity was noted for 48d, confirming the recent observations (25) that these two antibodies recognize different epitopes. It is interesting that the CD4 dependence of 48d binding was seen only with cell-associated variant viruses, suggesting that the non-CD4-bound multimeric structure of the variant gp120s is different from that of HXB2, thus possibly obscuring the 48d-binding site to a greater extent than is seen with HXB2 until CD4 is bound.

The results presented in this report draw us to the conclusion that neutralization escape of HIV-1 from natural human sera occurs through single amino acid substitutions that affect the binding of neutralizing antibodies at multiple sites on gp120. Numerous studies have illustrated interactions between envelope regions and how changes in one region can produce alterations in noncontiguous regions (5, 18, 24, 26, 27, 42, 51, 53). Thus, our conclusion is not unexpected. Moreover, the outcome of such changes probably includes the resistance to neutralization of primary HIV-1 isolates. In vivo immune selection pressures from polyclonal sera, leading to the sorts of single amino acid changes described here, could easily result in a generalized increase in resistance to neutralizing antibodies. It is not yet clear whether the mechanism for multiple escape involves a global change in the shape of gp120 or an alteration in the structure of gp120 multimers that form the surface spikes of HIV-1 virions. Nevertheless, our data suggest that the envelope proteins of HIV-1 are capable of subtle variation, presenting an almost limitless range of varying immunologic properties, which will defy categorization into definitive serotypes.

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